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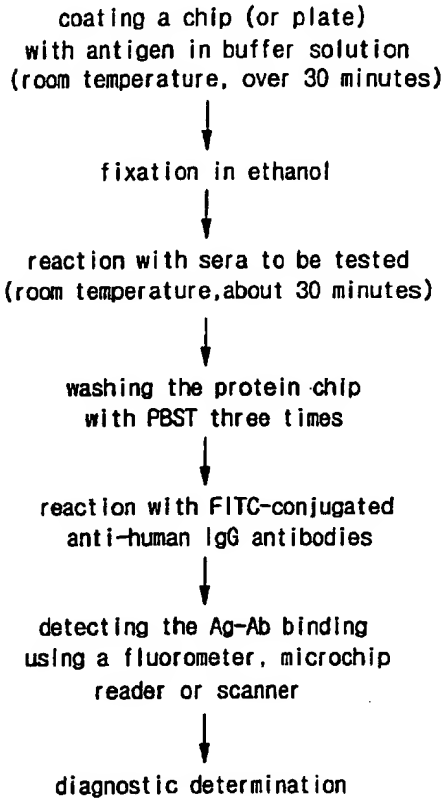
(54) Title: MULTIPURPOSE DIAGNOSTIC SYSTEMS USING PROTEIN CHIPS



(57) Abstract: The present invention provides protein chips on which high density of protein probe arrays are fixed, a method for manufacturing the protein chips, atomized diagnostic systems comprising the protein chips and the use thereof. The highly integrated structure of the protein chip makes a biochemical or an ummunological assay faster, suitable for automatization, precise and easy to handle. The usage of the protein chip encompasses clinical diagnosis, researches for the kinetics of enzymatic reactions and screening antagonists or ligands which bind to the interested receptors. In particular, the protein chip enables multipurpose diagnosis of various diseases for a number of patients even by a test.

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MULTIPURPOSE DIAGNOSTIC SYSTEMS USING PROTEIN CHIPS

Background of the Invention

The present invention relates to a protein chip which is prepared by fixing a plurality of probe spots on a solid micro substrate, wherein the probes are proteins or polypeptides capable of binding to target proteins in test samples. The present invention further relates to a method for manufacturing the protein chip, a method for diagnosing diseases of subjects and an automated diagnostic and/or analytic system using the protein chip. In particular, the automated diagnostic and/or analytic system comprises one or more protein chips of the invention, on which a number of probe spots are integrated in defined arrangement, a microarrayer performing a reaction between the probe and target proteins in test samples (e.g., sera of subjects), a reader for acquiring the signal of the reaction, and a means for transforming the signals into computer-readable data and analyzing the data. The protein chip of the invention combined with the diagnostic system can be used in analyzing such materials as an antigen comprised in samples quantitatively and qualitatively enabling multipurpose diagnoses of various diseases even with a small amount of samples for a number of subjects at a time, with a high throughput. Further advantages of the protein chip of the invention include feasibility for automation and rapidity of the diagnostic processes, and possibility of constructing profiles of specific diseases.

A biological chip or a biochip also referred to as a biological array is a new terminology introduced only recently. The biochip refers to a solid support substrate on which biological substances such as nucleic acids are fixed in a defined arrangement suitable for the purpose of use. As a well-known biochip, there is a DNA chip on which the fixed substance is

DNA fragments.

In this specification, the "substrate" (used in "solid substrate" or "micro substrate") refers to microplates or microchips, with small size and thin thickness, made of various materials. The principle of the biochip is based on the interaction of probe proteins immobilized on a micro-substrate and target proteins. For example, in the case of DNA chip, this interaction is complementary hybridization of oligonucleotides immobilized on the microchips and DNA present in test samples; and, in the case of a protein chip, protein-protein interaction such as antigen-antibody interaction or ligand-receptor binding.

The improvement of DNA sequencing technique has revealed nucleotide sequence of DNAs contained in a genome of many organisms from bacteria to a human and the completion of the Genome Project will give us more information about structures and functions of the genomes.

However, since it is so laborious and time-consuming to analyze the huge quantities of genetic information newly disclosed everyday through conventional techniques, more rapid and accurate techniques which enables to analyze many genes simultaneously are required.

Accordingly, DNA chips have been developed for genetic researches by combining conventional techniques in molecular biology with mechanical automation and electronically controlling techniques. "DNA chip" refers to a chip on which many DNA fragments are immobilized in a highly integrated manner for a gene analysis.

Conventional techniques employed for molecular biological researches, such as a southern blot, a northern blot, screening of mutant, and DNA sequencing, are applied only in a test for small size of samples. However, the development of DNA chips overcomes the limits in sample size, resulting that a number of genes can be analyzed on a large scale even by a test with a small quantity of sample. Also these features of DNA chips are advantageous for automatization.

As described above, DNA chips are expected to replace

conventional research techniques because of its rapidness and accuracy for accumulating and processing data, the convenience of operation and feasibility for automation. DNA chips will have a wide range of applications, such as in the functional analysis of genes, screening of cancer or disease-related genes (Yershov K. *et al.*, *Genetics* 93:4913-4918, 1996; Heller R.N. *et al., Proc. Natl. Acad. Sci.* USA 94:2150-2155, 1997; Derisi J. *et al., Nat. Genet.* 14:457-460, 1996), gene therapies, quarantines of animals or plants, examinations of food safety, development of new medicines (Winters M.A. *et al., Antimicrob. Agents Chemother.* 41(4):757-762, 1997), study of mutations (Ginot F., *Human Mutation* 10:1-10, 1997; Hacia G.H. *et al., Nat. Genet.* 14:441-447, 1996), DNA sequencings (Dramanac R. *et al., Electrophoresis* 13:566-573, 1992; Walraff G. *et al., Chetech February*, 22-32, 1997), tissue-inspections, identification of pathogenic microorganisms, and medical jurisprudence.

DNA chips are classified into 4 types below, according to their manufacturing method, especially the method for fixing oligonucleotides on their substrate:

1) A DNA chip of pin microarray type, wherein oligonucleotides are inlaid at a predetermined location by a pin-type microarrayer.

2) A DNA chip of ink jet type, wherein oligonucleotides in a cartridge are sprayed onto the chip by electronic forces.

3) A DNA chip of photolithographic array type, wherein oligonucleotide arrays are directly synthesized on the chip using photosensitive chemicals in a light-directed manner.

4) A DNA chip of electronic array type, wherein (-) charged DNA attaches to (+) charged materials coated on the chip.

Means for acquiring data from a DNA chip such as fluorescence reader (Shalon D. *et al., Genome Res.* 6:639-645, 1996), electronic signal reader (Sosnowski R.G. *et al., Proc. Nat. Acad. Sci.* USA 94:1119-1123, 1997) have also been developed.

Though various techniques for the application of DNA chips for

genetic researches have been developed or are being developed, research on protein chips suitable for constructing an automated system for diagnosing various diseases is still at its initial stages.

Methods for diagnosing diseases developed so far include the use of reagents for blood cell agglutination, radioimmunoassay (RIA), enzyme immunoassay (EIA), chemiluminescence immunoassay (CLIA), etc. These conventional diagnostic methods comprise the steps of: immobilizing antigens, incubating the immobilized antigens with antibodies in the sera or other samples of subjects, and detecting the antigen-antibody interaction with secondary antibodies labeled with radioactive chemicals, enzymes or fluorescence dyes, etc.

Since conventional methods for diagnosis based on protein-protein interaction are applicable to only small quantity of samples at a time, as in genetic analysis, and require lots of time, labors and cost, they are not suitable for diagnosing multiple diseases of many people or automatizing the diagnostic processes.

The method of CLIA, comparatively well automated among the above methods, since in early 1990s when the method was first developed, gradually has replaced the EIA method including enzyme-linked immunosorbent assay (ELISA). However, difficulties in development of chemiluminescence materials and an automatic system therefor are still obstacles for CLIA to be suitable for wide range of its application to the prevention and the therapy of pathogenic diseases through early diagnosis.

Accordingly, it has been desired to develop a protein chip which can embody the combination of highly advanced technology in mechanics and electronics with molecular biological techniques and an automated diagnostic system using it, for the prevention and the therapy of pathogenic diseases.

A protein chip is different from a DNA chip in that the substance immobilized on the substrate is protein rather than DNA; it requires multiple-step reactions (such as antigen-first antibody interaction, primary

antibody-secondary antibody interaction and washing between the reaction steps); and, therefore, different method and condition for manufacturing a protein chip and different reaction conditions are needed as well as delicate controlling technique.

More specifically, many factors have to be considered for manufacturing protein chips. Firstly, antigenic proteins or polypeptides to be fixed on the chips have diverse electrical properties according to their kinds and structures unlike DNA having (-) charge commonly. Secondly, the proteins or polypeptides are relatively large in size compared with a DNA probe having the size ranging from 15-25 base pairs to approximately 500 base pairs. Thirdly, the antigenic proteins or polypeptides immobilized on chips must retain their antigenic conformation necessary for binding to antibodies in test samples.

Accordingly, a method for fixing proteins to the substrate is inevitably different from that for fixing DNA. Further, contrary to the DNA chip containing DNA fragments of similar properties, the protein chip should contain various antigenic proteins on a substrate, and, accordingly, it is required that the antigenic proteins are immobilized at an optimum condition determined by considering the optimum immobilization condition for each antigenic protein. Moreover, for the correct reaction on a micro substrate, a high accuracy in the reaction locations is required for a protein chip which employs an immunoassay method requiring multi-step reactions. Likewise, different control programs from those used for DNA chips are required to be developed for the multi-step reactions of a protein chip.

As discussed above, the research of protein chips, for which many factors are to be considered as compared to DNA chips, are at a preliminary stage due to difficulties entangled in the development thereof.

However, since protein chips are based on protein-protein interactions, they have a superior utility in diagnosing disease to DNA chips which diagnose only genetic diseases or cancers through the

detection of genetic abnormality. Protein chips have a wide range of applicability, e.g., in the diagnoses of various kinds of metabolic diseases such as virus- or bacteria-infected diseases, search for antagonists to various kinds of receptors by using a receptor-ligand binding reaction, and researches on the enzyme-substrate interactions and screening for the inhibitors of an enzyme by employing the chip wherein the enzyme is immobilized thereon. In addition, in the aspect of automation, early construction of a system therefor is possible by using the previously developed DNA chip analyzers.

Summary of the invention

It is an object of the present invention to provide a protein chip which can analyze many samples at one time, has a high accuracy and is suitable for an automated analytical or diagnostic system. Another object of the present invention is to provide a protein chip having a wide range of applicability such as diagnoses of various kinds of metabolic diseases and viral or bacterial infections, and screening of antagonists useful for the development of new medicines, different from a DNA chip used for the genetic analysis or diagnoses of diseases caused by genetic abnormalities.

A further object of the present invention is to provide an optimum method for preparing a protein chip, conditions for the method being inevitably different from that for a DNA chip since the probe materials immobilized on the two kinds of chips are different from each other.

Additional object of the present invention is to provide biological and chemical materials that are necessary for constructing a protein chip useful for diagnosing various diseases.

A still another object of the present invention is to provide a protein chip that can perform simultaneously the diagnoses of plural diseases in a subject, of one disease in plural subjects, and of plural

diseases in plural subjects.

A still further object of the present invention is to provide an automated system where mass-diagnosis are made by using said protein chip and a database is constructed from the data acquired in a short time from said mass-diagnoses for use in the diagnoses, preventions, and treatment of diseases in a human body.

To accomplish said purposes, the present invention provides a protein chip for mass-diagnosis, which comprises a micro solid substrate on which a plurality of spots of probe proteins are fixed in a defined arrangement, wherein,

1) the probe proteins are selected from the group consisting of antigens, receptors and enzymes;

2) the probe proteins are fixed on the micro solid substrate via bonds between amino groups of the probe proteins and functional groups of chemicals coated on the substrate;

3) the probe proteins are capable of binding to target proteins in a test sample; and

4) the quantity of the probe proteins per spot is minute, e.g., several to dozens pg, thereby reducing the required amount of a sample to a minimum.

The present invention further provides a method for manufacturing a protein chip containing a micro solid substrate on which a plurality of spots of probe proteins are fixed in a defined arrangement, which comprises the steps of:

1) arraying mixtures of a coating buffer and one or more kinds of probe proteins at predetermined locations on the micro solid substrate, with the quantity of the proteins per spot in a minute amount, e.g., several to dozens pg;

2) immobilizing the probe proteins by incubating the substrate at room temperature;

3) fixing the probe proteins on the substrate by immersing the

substrate in 100% ethanol; and

4) drying the substrate obtained in step 3).

The present invention also provides a method for analyzing target proteins present in test samples quantitatively or qualitatively, which comprises the steps of:

1) reacting a test sample with the inventive protein chip;

2)washing the protein chip;

3) reacting the protein chip obtained in 2) with fluorescence-conjugated secondary antibodies specific for a target protein, said target protein being capable of binding a probe protein fixed on the protein chip; and

4) detecting the reaction signals with a fluorescence microscope or a microchip reader.

Moreover, the present invention also provides an automated system for diagnosing a plurality of diseases in plural subjects comprising:

1) a protein chip containing a micro solid substrate on which a plurality of spots of probe proteins are fixed in a defined arrangement;

2) a first microarrayer capable of arraying one or more probe proteins in plural spots on the protein chip;

3) a second microarrayer controlled to perform sequentially allotting test samples exactly to the locations at which the probe proteins are fixed on the protein chip, washing the protein chip after reaction, and adding secondary antibodies to react with target proteins in the test samples; and

4) a fluorescence microscope or a micro chip reader for detecting the reaction between the probe proteins and the target proteins.

The present inventors have endeavored to construct a multi-purpose automated diagnosing system by developing a protein chip capable of mass diagnosing various viral diseases, and, consequently, have discovered that a clear discrimination between positive and negative serum samples are exhibited when antibodies in the samples are monitored by

employing a protein chip prepared via coating various antigens on a solid substrate. The present inventors have accomplished the present invention by preparing said protein chip by using an automated microarrayer system which has been employed for preparing a DNA chip and confirming the correct discrimination between positive and negative samples by the protein chip with a high speed fluorescence scanner.

Brief description of the drawings

Fig. 1 shows the genome of HIV used in the present invention and the cloning regions of gag and *env* antigens therein;

Fig. 2 represents the genome of HCV used in the present invention and the cloning regions of antigen therein;

Fig. 3 exhibits schematic view of a process of an antibody-antigen reaction using the protein chip of the present invention;

Fig. 4 displays a result of an antibody-antigen reaction for hepatitis B virus(HBV) which is analyzed using the protein chip of the present invention(a: a positive serum spread on the surface of slide and b: a negative serum spread on the surface of slide);

Fig. 5 illustrates a result of an antibody-antigen reactions for human immunodeficiency virus(HIV) which is analyzed using the protein chip of the present invention(a: a positive serum spread on the surface of slide and b: a negative serum spread on the surface of slide);

Fig. 6 depicts a result of an antibody-antigen reaction for hepatitis C virus(HCV) which is analyzed using the protein chip of the present invention(a: a positive serum spread on the surface of slide and b: a negative serum spread on the surface of slide);

Fig. 7 describes a schematic view of a FITC conjugated antibody which is spread using a microarrayer;

Fig. 8 is a photograph of fluorescence microscope of FITC conjugated antibody which is spread using microarrayer;

Fig. 9 shows a schematic view of the HCV antigen which is spread using a microarrayer;

Fig. 10 represents a result of the antibody-antigen reactions for hepatitis C virus(HCV) which is analyzed using the protein chip of the present invention(a: positive serums spread on the surface of slide and b: negative serums spread on the surface of slide(beginning with stock solution and solutions diluted by factors of 10^{-1} and 10^{-2} in a and b, respectively));

Fig. 11 exhibits a result of the specificity for HCV which is analyzed using the protein chip of the present invention(a: positive serums spread on the surface of slide and b: a negative serum spread on the surface of slide);

Fig. 12 displays a result of the specificity for HIV which is analyzed using the protein chip of the present invention(a: positive serums spread on the surface of slide and b: a negative serum spread on the surface of slide); and

Fig. 13 compares the sensitivities of the antigen-antibody reactions for HCV and HIV which is analyzed using the protein chip of the present of invention with those of conventional ELISA reaction(a: the sensitivity of antibody-antigen reaction for HCV and b: the sensitivity of antigen-antibody reaction for HIV(from stock solution to the diluted solution by 2 folds of positive serum and negative serum).

Detailed description of the invention

The protein chip of the present invention comprises a micro solid substrate on which a plurality of spots of probe proteins are fixed in a defined arrangement, wherein, 1) the probe proteins are selected from the group consisting of antigens, receptors and enzymes, 2) the probe proteins are fixed on the micro solid substrate via bonds between amino groups of the probe proteins and functional groups of chemicals coated on the

substrate, 3) the probe proteins are capable of binding to target proteins in a test sample; and 4) the quantity of the probe proteins per spot is minute, e.g., several to dozens pg. The protein chip of the present invention is characterized by its capability of performing simultaneous analysis or diagnosis on a plurality of samples at a time.

As used herein "probe protein" refers to the proteins that are regularly arranged and immobilized on a micro solid substrate and can bind to the target proteins to be detected. For instance, the probe protein may be selected from the group consisting of antigens, antibodies, receptors, enzymes etc. Specifically, in case of using antigens as probe proteins, it is possible to detect the presence of antibodies in a sample, thereby making it possible to diagnose various diseases. Further, in case when receptor proteins are used as probe proteins, it is possible to examine ligands which bind to the receptor proteins and inhibitors of said receptor-ligand binding. Alternatively, in case when enzymes are used as probe proteins, the inventive protein chip can be applied for the study on the enzyme-substrate reactions, and to screen for inhibitors of enzymatic activity.

Preferable examples of the probe proteins include antigenic proteins originated from unicellular organisms inclusive of virus, bacteria and fungi, animals, and plants. In a preferred embodiment, it has been confirmed that by using the inventive protein chip prepared by employing one or more of the antigenic proteins originated from hepatitis B virus (HBV), human immunodeficiency virus(HIV) or hepatitis C virus(HCV), it is possible to diagnose simultaneously various diseases such as hepatitis B, AIDS, hepatitis C, etc.

As used herein, "target protein" refers to a high molecular-weight compound that is included in samples to be tested and can bind the probe proteins immobilized on the protein chip. Examples of the target proteins include antibodies for the disease to be diagnosed in case when antigenic proteins are used as probe proteins; antigens for the disease to be diagnosed in case when antibodies are used as probe proteins; various ligands or

ligand-candidates in case when the receptor proteins are used as probe proteins; and substrates or inhibitor candidates in case when enzymes are used as probe proteins, and, in addition, various kinds of polysaccharides, carbohydrates, and compounds can also be used as targets. Preferable targets are those substances whose binding to various probe proteins can be regulated by changing physicochemical conditions.

The substrate of said protein chip refers to a solid plate on which a plurality of spots of immobilized probe proteins are arranged regularly, and it is preferred that surface of the substrate is coated.

The solid substrates may be made of glass; modified silicone; or commonly used polymers or gels such as tetrafluoroethylene, polystyrene, and polypropylene. Preferably, the surface of the substrate is coated for the immobilization of the probe proteins with a chemical selected from the group consisting of polymers, plastics, resins, carbohydrates, silica, silica derivatives, carbon, metals, inorganic glasses and membranes.

It is preferred that the chemicals used for coating the substrate have functional groups for binding with amino groups on the probe proteins, said functional groups having preferably an alkyl group, e.g., aminoalkylsilane.

The substrate serves as a support for immobilizing the probe proteins and provides a space wherein the binding reactions between immobilized probe proteins and the targets in the samples occur. The size of the substrate, and the location, size, and shape (for example, dot or line) of the immobilized probe proteins may be varied with the purpose of the analysis, devices including automatic liquid-handling apparatus and reader, etc.

Probe proteins are arranged in a predetermined defined area on the micro solid substrate, and preferable but not limitative example of said predetermined defined area is a circular spot having a diameter ranging from 150 to 1000μm.

Preferable examples of said solid substrate include a tetragonal plate having a size of about 2.5 cm × 7.5 cm or a circular disk having a

diameter of about 2-10 cm, and about 100-10,000 spots can be arranged per protein chip. The spots are arranged in plural columns and rows or they are arranged around the circumference of the round disk. Further, a multi-purpose protein chip can be prepared by dividing the substrate into one or more sectors and immobilizing proteins of a kind in each sector, said proteins in a sector being different from those in other sectors.

For instance, in case when the antigenic proteins immobilized in each of the divided sectors on the substrate are responsible for each of various diseases, it is possible to diagnose the infection of said diseases by one analysis using a serum sample from one subject. Moreover, in case when samples from plural subjects are applied respectively to different sectors, it is possible to diagnose the infection of various diseases in plural subjects.

In order to accelerate the immobilization of the probe protein by strengthening the binding of the probe proteins and the substrate, it is preferable to immerse the probe protein-coated chip in ethanol solution having a concentration of more than 98%, preferably, 100%.

On the other hand, the present invention also provides a method for preparing a multipurpose protein chip having the features as described above. The inventive method comprises the steps of: 1) arraying mixtures of a coating buffer and one or more kinds of probe proteins at predetermined locations on a micro solid substrate, with the quantity of the proteins per spot ranging from several to dozens pg; 2) immobilizing the probe proteins by incubating the substrate at room temperature; 3) fixing the probe proteins on the substrate by immersing the substrate in 100% ethanol; and 4) drying the substrate obtained in step 3).

As a coating buffer for the above process, sodium phosphate buffer or sodium carbonate buffer may be used and, preferably, about 10mM sodium phosphate buffer or 50 mM sodium carbonate buffer may be used. The kind and concentration of a coating buffer may be selected appropriately considering the kind and property of the probe protein to be

immobilized on the substrate. For instance, it is preferable to use sodium phosphate buffer for antigens of HBV; and sodium carbonate buffer, for the antigens of HIV or HCV.

It is preferable to use an automatic microarrayer or liquid-handling devices corresponding thereto in order to arrange and immobilize the probe proteins on the substrate since they allow rapid and massive production or processing of the protein chips. Currently, a microarrayer suitable for the manufacture of a protein chip has not yet been developed, and, accordingly, a microarryer for manufacturing a DNA chip, e.g., GMS 417 Arrayer™ (Genetic MicroSystems Industry) may be provisionally used in the automated manufacturing process of the present invention.

The method for analyzing quantitatively or qualitatively a target protein in a sample using the inventive protein chip comprises:

1) reacting a sample with the inventive protein chip;

2) washing the resulting protein chip;

3) reacting the protein chip obtained in 2) with fluorescence-conjugated secondary antibodies having specificity for target protein, the target protein being capable of binding a probe protein fixed on the protein chip; and

4) detecting the resulting fluorescence using a fluorescence microscope or microchip reader.

An embodiment of the method for analyzing a target protein may employ a protein chip which contains two or more kinds of antigen proteins of different diseases, each antigen protein being fixed on a separate portion of the micro solid substrate, and a serum sample taken from a subject to detect the resulting fluorescence using a fluorescence microscope or microchip reader, by which it can be determined whether subject carries such antigens.

Another embodiment of the method for analyzing a target protein may employ a protein chip which contains a kind of antigen proteins fixed on the micro solid substrate and two or more serum samples taken from

plural subjects to detect the resulting fluorescence, thereby determining the subject's infection of the disease.

Further, the method for analyzing a target protein may employ a protein chip which contains two or more kinds of antigen proteins generating different diseases, each antigen protein being fixed in array on the micro solid substrate, and two or more serum samples taken from plural subjects, thereby determining the subject's infection of such diseases. Such a method gives plural groups' profiles of various diseases at a time.

Binding of a probe protein fixed on the protein chip to a target protein contained in the sample may be detected by using any the conventional assay methods, e.g., radioimmuno assay(RIA), enzymeimmuno assay(EIA) or chemiluminiscenceimmuno assay(CLIA), among which a florescence detecting system is preferred to process automatically a large number of protein chips in a short time. If an automated reader, e.g., fluorescence-detecting biochip reader, may be used in detecting the binding of a probe protein to a target protein, it would be possible to industrialize directly without development of additional fluorescence-detecting device. An embodiment of the present invention describes the method for detecting the antigen-antibody reaction on the protein chip using the fluorescence-conjugated anti-human immunoglobulin G, particularly by reading a large number of protein chips automatically using high-speed scanner GMS 418 Array Scanner^{TM}(Genetic Microsystems industry) which has been used in analyzing DNA chip.

Use of the automated liquid-handling device and biochip reader may allow to manufacture and read a large number of protein chips at a time. The protein chip thus manufactured is particularly suitable in making a database of disease profiles obtained from individuals or groups thereof.

The present invention further provides an automated diagnosis system for acquiring plural subjects' profiles for plural diseases in a short

time, which comprises: the inventive protein chip; a first microarrayer for arranging automatically one or more kinds of probe proteins in plural spots on the protein chip; a second microarrayer for adding accurately a sample to target protein-fixed location of the protein chip, the second miroarrayer being controlled to perform sequentially allotting a sample to the location, washing the resulting reaction mixture and adding secondary antibodies thereto; and a fluorescence microscope or microchip reader for detecting the binding of probe protein to target protein.

Preferably, the automated diagnosis system further comprises a computerized device for making database of disease profiles with the data acquired by using the fluorescence microscope or microchip reader.

The method for quantitative or qualitative analysis of target proteins is useful in diagnosing various diseases, preferably infectious diseases. Compared with the ELISA or CLIA method, the inventive diagnosis system using the protein chip is conducted by reading even small amount of antigen(10^{-6} to 10^{-5} orders of amount) and antibodies and detecting the resulting fluorescence, and, therefore, it is very economic and has high sensitivity and specificity. Specially, ELISA method requires at least 100 ng of antigens per well whereas the inventive diagnosis system requires at least 1 pg of antigens per spot. Therefore, the inventive diagnosis system examines plural blood samples using even a small amount of antigens. Further, the microarray system comprising automated liquid-handling device and reader may reduce necessary steps, thereby preventing waste of time, labor and resource, and may provides a good diagnosis only by a simple fluorescence-detecting step, thereby being effective in early diagnosis for various infectious diseases and prevention thereof.

Another embodiment of the present invention describes an immunological diagnosis using a protein chip which contains Hepatitis B virus(HBV), human immunodeficiency virus(HIV) or Hepatitis C virus(HCV) antigens with positive and negative serum samples against each antigen(Fig. 3). Antigen-antibody reaction was detected in all

positive serum samples but not at all in negative serum samples(Figs. 4 to 6 and 10). Fluorescein isothiocyanate(FITC)-conjugated antibody could sufficiently be detected with the biochip reader GMS 418 Scanner™(Fig. 8), which suggested that the manufacturing time for a suitable detector can be reduced. Further, specificity and sensitivity of HCV or HIV antigen-antibody reaction were examined and the results show that specificity and sensitivity thereof were excellent(Figs. 11 to 13). These results suggest that the inventive method for analyzing a target protein and the inventive system comprising the protein chip are useful in diagnosing various infectious diseases.

Comparisons of the inventive automated diagnosis system with ELISA and CLIA are shown in Table 1.

Table 1

Items	ELISA method	CLIA method	Inventive automated system using protein chip
Sample-processing portion	96-well plate ($12\times8cm^2$)	Cuvette-type tube	slide glass ($2.5\times7.5\ cm^2$)
No. of samples that can be tested per plate	96 or less	60	100 - 40,000
Reaction temperature	37℃	37℃	Possible at room temperature
Simultaneous examiniation	possible for certain items	possible for certain items	possible for all items
Required volume of sample	100 – 200$\mu\ell$	10 – 200$\mu\ell$	10 – 100 pl
Processing time (h)	4 – 5	2 – 3	less than 1
Processing rate(samples/h)	60	180	40,000 or less

*For example, GMS 417 Arrayer™ for DNA chip, can examine 60 chips simultaneously and the microarrayer can place 4 spots per sec. and give results using the analyzer having a capacity of reading 30 lines per sec.

As can be seen from Table 1, the inventive automated diagnosis system of the present invention using the protein chip is more efficient in terms of time, labor and resources than ELISA or CLIA method. That is, it is possible to diagnose simultaneously tens of diseases using a subject's blood sample, a viral disease using hundreds of subjects' blood samples or tens of diseases using hundreds of subjects' blood samples. Consequently, the automated diagnosis system can construct disease profiles of individuals constituting a specific group into database, which can be used actively in managing disease and preservation of health at nation level.

The following Examples are intended to further illustrate the present invention without limiting its scope.

Preparation Example 1: Preparation of viral antigen for coating protein chip

HBV antigens were purchased from OEM CONCEPTS(#H6-V19, USA) and, HIV and HCV antigens were prepared as follows.

In order to prepare HIV antigens, HIV genome(GenBank Accession Number U26942) was subjected to polymerase chain reaction(PCR) to amplify polynucleotides encoding capsid protein p24 of gag protein and extracellular domain gp41of envelope protein. Each PCR product was cloned to vector pCRII(Invitrogen, Netherland) and BglII/BamHI fragment of the recombinant vector was inserted at BglII/BamHI sites of vector pMAL-c2(New England Biolabs Inc ;NEB, USA) to obtain an expression vector. Primers and reaction condition used in the PCR were shown in Table 2.

Table 2

Amplified gene	Primer	Nucleotide sequence	SEQ ID NO.
HIV p24	Forward	AGATCTCCTATAGTGCAGAACCTCCA	3
	Reverse	GGATCCCAAAACTCTTGCTTTATG	4
HIV gp41	Forward	AGATCTAGAGCAGTGGGAATAGGA	5
	Reverse	GGATCCATTCCACAAACTTGCCCA	6
*PCR condition: denaturation(94℃, 1 min.) → annealing(55℃, 1 min.) → extension(72℃, 90 sec.) /total 30 cycles			

The expression vector thus obtained was introduced into the *E. coli* Top10F' to overexpress the antigen proteins. Then, the antigen proteins were purified by manufacturer's instruction and the purified antigen proteins were used as probe proteins of protein chip(Fig. 1).

As HCV antigens, the polynucleotide(SEQ ID NO: 1) encoding NS3 antigen and the polynucleotide(SEQ ID NO: 2) encoding NS3core fusion antigen of Korean HCV were selected. NS3 antigens were prepared by conducting PCR; cloning the PCR product into vector pCRII; inserting the NdeI/EcoRI fragment of the resulting recombinant vector at NdeI/EcoRI sites of T7-tagged pET-17b vector(Novagen, USA); transforming *E. coli* Top10F' with the resulting expression vector to overexpress antigens; transforming the resulting transformant with BL21; purifying antigens according to manufacturer's instructions. The purified antigens were used as probe proteins of protein chip. NS3 fusion antigens were prepared by conducting PCRs to amplify polynucleotides encoding core and NS3 antigens, respectively; cloning the polynucleotides into vector pCRII, respectively; inserting fragments of the recombinant vector

at BglII/XbaI and BglII/BamHI sites of expression vector IciA vector; transforming *E. coli* Top10F' with the resulting recombinant vector to overexpress antigens; transforming the resulting transformant with BL21; purifying the antigens according to manufacturer's instructions. The purified antigens were used as probe proteins of protein chip(Fig. 2). The PCR primers for amplifying the polynucleotide encoding the NS3 antigen and NS3core antigen of HCV are shown in Table3. PCR was performed as like the amplification of HIV antigen gene.

Table 3

Amplified gene	Primer	Nucleotide sequence	SEQ ID NO.
HIV24	Forward	AGATCTCATATGGACTTCATACCCGTTG	7
	Reverse	GAATTCTCTAGACTAACATGTGTTACAGTCG	8
	Forward	AGATCTATGCGGTCTCCGGTCTTC	9
	Reverse	GGATCCTTTTAGCCGTATGACACA	10
HIVgp41	Forward	AGATCTATGAGCACAAATCCTAAA	11
	Reverse	TCTAGACTAGAGGTCGGCGAAGCCGCA	12

Preparation Example 2: anti-serum for detecting antigen

For detecting HBV antigen, 10 HBV-positive and 2 HBV-negative serum samples provided by Institute of molecular biology &

genetics of Seoul National University and, HBV-positive and HBV-negative control serum samples contained in ELISA kit of Green Cross Co. were used. For detecting HIV antigen, 10 HIV-positive serum samples provided by medical college of Seoul National University, National institute for Health, Central Medical Center and Yonsei University, and 5 HIV-negative serum samples provided by Institute of molecular biology & genetics of Seoul National University were used. For detecting HCV, 12 HCV-positive and 8 HCV-negative serum samples provided by Green Cross Co. were used. To prevent infection by HIV, the HIV-positive serum samples were diluted by 2-fold and then heat-inactivated at 50℃ for 2 hours before use.

Example 1: Detection of HBV antigen-antibody reaction using protein chip

HBV antigen-antibody reaction was conducted as shown in Fig. 3.

(1-1) Manual preparation of protein chip

HBV antigens obtained in Preparation Example 1 were diluted to a final concentration of 100 ㎍/㎖ with coating buffer(10mM sodium phosphate, pH 7.6). Each 1 ㎕ of diluted antigen solution was added on the surface of aminoalkylsilane-coated microscope slide glasses(#S4651, Sigma Diagnostics, USA). The resulting slide glasses were kept at room temperature, and then, the coated antigens thereon were fixed by immersing the antigen-coated protein chip in 100% ethanol to reinforce bonding of amino group of antigen to alkyl group on the slide glasses and drying the resulting slide glasses completely.

(1-2) Antigen-antibody reaction

Each 1 ㎕ of HBV-positive and HBV-negative serum samples was added to the surface of the antigen-coated slide glasses. As a control, 1 ㎕ of dilution solution(sodium phosphate buffer(PBS) containing 0.25 % Tween® 20 and 0.5 % bovine serum albumin) was used. The resulting slide glasses were incubated in chamber at room temperature for 30 min., washed three times with PBST(PBS containing 0.05 % Tween® 20) and dried. 1㎕ of the fluorescent-conjugated anti-human IgG antibody(#31529, Pierce Co., USA) were added to each spot of the slide glasses and incubated at room temperature for 15 min. in the chamber. After completion of the reaction, the slide glasses was washed three times with PBST and dried. Each fluorescence of the slide glasses was detected by fluorescence microscope and photographed.

The HBV antigen-antibody reaction(Fig. 4) was repeated four times. All of the positive serum samples and positive control solution showed intensive fluorescence while the negative serum samples and negative control solution did not show any fluorescence and the control dilution solution did not.

Example 2: Detection of HIV antigen-antibody reaction using protein chip

The procedure of Example 1 was repeated except that HIV antigen and anti-HIV serum samples obtained in Preparation Example 1 and 2, respectively, were used in place of HBV antigen and anti-HBV serum samples.

HIV antigens obtained in Preparation Examples 1 and 2 were diluted to a final concentration of 100 ㎍/㎖ with coating buffer(50mM sodium carbonate, pH 9.6). HIV antigen-antibody reaction(Fig. 5) was

repeated three times similarly with the above HBV antigen-antibody reaction. As the result, all positive serum samples showed evident fluorescence, while the negative serum samples and control did not show any fluorescence. Fluorescence intensities of the positive serum samples were materially different to those of the negative serum samples, and the coincident results were shown in ELISA.

Example 3: Detection of HCV antigen-antibody reaction using protein chip

HCV antigens obtained in Preparation Examples 1 and 2 were diluted to a final concentration of 100 $\mu g/m\ell$ with coating buffer(50 mM sodium carbonate, pH 9.6) . The procedure of Example 1 was repeated except that HCV antigens and anti-HCV serum samples obtained in Preparation Examples 1 and 2 were used in place of HBV antigens and anti-HBV serum samples.

As the result of reacting HCV antigen with positive or negative serum samples on the protein chip(Fig. 6), the positive serum samples showed intensive fluorescence while the negative serum samples and control did not show any fluorescence.

Example 4: Reading of FITC-conjugated antibody using high-speed fluorescence scanner

Using high-speed fluorescence scanner for reading DNA chip, GMS418 Array Scanner™(Genetic Microsystems, GM200), fluorescence of fluorescence-conjugated anti-human IgG (#31529,Pierce, USA) bound to antigen on the slide glass was determined.

Four sets of antibodies were added in a spot number of 400 spots/set using DNA chip-manufacturing device GMS 417 Arrayer™(Genetic MicroSystems, GM100)(Fig. 7). Fluorescence was

read at 532 nm in the flying objective microscope mode, and the resulting data were analyzed using the expression data analysis software Imagene™(Genetic MicroSystems, version 2.0).

Result of analyzing the slide thus manufactured using the high-speed fluorescence scanner is shown in Fig. 8, which suggest that the scanner known to only recognize DNA-staining fluorescence dyes Cy3 and Cy5 can read FITC conjugated antibody sensitively.

Example 5: Construction of HCV protein chip using microarrayer and antigen-antibody reaction

A protein chip for HCV antigen was constructed using GMS 417 Arrayer ™f as shown in Fig. 9.

HCV antigen having an initial concentration of 100 $\mu g/\mu \ell$ was serially diluted to a dilution factor of 10^{-5}. The diluted antigen solutions were added to a 96 well microplate and the microplate was set in a microarrayer. Each 10 pℓ of the diluted antigen solutions were spotted for three times in an array mode using 4 pins and rings to construct a protein chip including 4 lanes having 18 spots per lane, wherein the interval between spots was 375 μm; the interval between lanes, 0.9 mm; a diameter of spot, 150 μm; and unit amount of antigen based on the initial concentration, 1 pg/mℓ.

The protein chip was reacted with antibodies according to the procedure shown in Fig. 3, provided that the antibodies spotted on a parafilm was adhered closely to a surface of the slide glass rather than reacted with each spot, considering the size of the spots and the intervals between them.

After the completion of the antigen-antibody reaction, positive or negative reaction was confirmed by a fluorescence microscope and photographed (Fig. 10).

As can be seen from Fig. 10, the result showed a greater difference depending on the dilution factor of the antigen. The HCV antigens diluted by factor of up to 10^{-1} exhibited a positive reaction for HCV serum of a patient and the HCV antigens diluted by a factor of 10^{-2} or more showed little fluorescence for the serum of a HCV patient. This result was considered to be caused by the fact that a unit amount of antigen based on the initial concentration, i.e., 1 pg, was too small and it is concluded that the concentration of antigen to cause a detectable antigen-antibody reaction on a protein chip is minimum 10 $\mu g/m\ell$, and suitably more than 100$\mu g/m\ell$. On the other hand, the negative serum sample did not show any fluorescence regardless of the dilution factors.

Example 6: Specificity of antigen-antibody reaction for HCV protein chip constructed by using the microarrayer

A protein chip for HCV antigen was constructed using GMS 417 Arrayer^{TMf} as shown in Fig. 7, and its reactivity for various samples were examined.

300 $\mu g/m\ell$ of HCV antigen was added to a 96 well microplate and the microplate was set in a microarrayer. HCV antigen was spotted on a slide glass(2.5×7.5 cm^2) (or chip) in an array mode using 4 pins and rings to construct a protein chip including 4 sets having 400 spots per set(see Fig. 7). 10 $p\ell$ of antigen was added per spot and, accordingly, 3 pg of antigen was used per sample.

The protein chip prepared above was reacted with 12 positive and 8 negative sera according to the procedure of Fig. 3. Due to some technical problems, one antibody was reacted per set and the antibodies spotted on a parafilm was adhered closely to a surface of the slide glass in order to apply the antibodies uniformly.

After the completion of the antigen-antibody reaction, the chip

was read at 532 nm with a flying objective microscope and the data obtained therefrom were analyzed using Imagene™(Genetic MicroSystems, version 2.0), a software for analyzing the expressed data (see Fig. 11).

As can be seen from Fig. 11 showing a result using one positive serum and one negative serum, clear positive and negative reactions were exhibited in the positive and negative sera, respectively. Similar results were obtained in all of the 12 positive and 8 negative sera. From this result, it is confirmed that the protein chip constructed using the microarrayer has a sensitivity enough to replace the conventional ELISA diagnostic reagents.

Example 7: Specificity of antigen-antibody reaction for HIV protein chip constructed by using the microarrayer

A protein chip for HIV antigen was constructed using GMS 417 Arrayer™f as shown in Fig. 7, and its reactivity with various samples were examined.

300 μg/mℓ of HIV antigen was added to a 96 well microplate and the microplate was set in a microarrayer. HCV antigen was spotted on a slide glass(2.5×7.5 cm^2) (or chip) to construct a protein chip including 4 sets having 400 spots per set(see Fig. 7). 10 pℓ of antigen was added per spot and, accordingly, 3 pg of antigen was used per sample.

The protein chip prepared above was reacted with 10 positive and 5 negative sera according to the procedure of Fig. 3. Due to some technical problems, one antibody was reacted per set and the antibodies spotted on a parafilm was adhered closely to a surface of the slide glass in order to apply the antibodies uniformly.

After the completion of the antigen-antibody reaction, the chip was read at 532 nm with a flying objective microscope and the data obtained therefrom were analyzed using Imagene™(Genetic MicroSystems,

version 2.0), a software for analyzing the expressed data (see Fig. 12).

As can be seen from Fig. 12 showing a result using one positive serum and one negative serum, clear positive and negative reactions were exhibited in the positive and negative sera, respectively. Similar results were obtained in all of the 10 positive and 5 negative sera. From this result, it is confirmed that the protein chip constructed using the microarrayer has a sensitivity enough to replace the conventional ELISA diagnostic reagents.

Example 8: Sensitivities of antigen-antibody reactions for HCV and HIV protein chips constructed by using the microarrayer

A protein chip for HCV and HIV antigens was constructed using GMS 417 Arrayer^{TMf} as shown in Fig. 7, and its sensitivity for the antibodies in blood samples was examined and compared with that of the conventional ELISA method

Each 300 $\mu g/m\ell$ of HCV and HIV antigens was added to a 96 well microplate and the microplate was set in a microarrayer. Antigens were spotted on a slide glass(2.5×7.5 cm^2) (or chip) to construct a protein chip including 4 sets having 400 spots per set(see Fig. 7). 10 $p\ell$ of antigen was added per spot and, accordingly, 3 pg of antigen was used per sample. The protein chip prepared above was reacted with positive and negative sera according to the procedure of Fig. 3. One positive serum and one negative serum were adopted for HCV and HIV antigens, respectively, and they were subjected to serial double dilutions. Due to some technical problems, one antibody was reacted per set and the antibodies spotted on a parafilm was adhered closely to a surface of the slide glass in order to apply the antibodies uniformly.

After the completion of the antigen-antibody reaction, the chip was read at 532 nm with a flying objective microscope, the data obtained

therefrom were analyzed using Imagene™(Genetic MicroSystems, version 2.0), a software for analyzing the expressed data (see Fig. 12), and intensity of the fluorescence was determined for each set.

One the other hand, conventional ELISA method was carried out by employing HCV and HIV antigens having a concentration of 2.5㎍/㎖. Each 100 ㎕ of antigens were added to a 96 well plate and the plated was allowed to stand at room temperature for more than 12 hours. The reaction was terminated with 0.5% BSA(in PBS) for 1 hour and the positive and negative sera were added thereto. A positive serum and a negative serum were subjected to the serial two-fold dilution with a sample dilution solution, respectively. Each 100 ㎕ of the diluted sera was added to each well and reacted in 37℃ thermostat for 2 hours. When the reaction was completed, the plate was washed five times with the same washing solution used for the protein chip. An anti-human IgG-HRP(horseradish peroxidase) conjugate was diluted to 1/10,000 dilution and 100 ㎕ of the dilution was then added to the well and reacted in 37℃ incubator for 1 hour. After the reaction, wells were washed five times with a washing solution and 100 ㎕ of tetramethylbenzidine (TMB) as a substrate solution was added thereto. The mixture was reacted at room temperature for 30 minutes and the reaction was terminated with a stopping solution (1N sulfuric acid solution). When the reaction was completed, absorptions were measured at 450 nm with an ELISA reader.

The analyses results using the protein chip and ELISA were represented as the S/CO ratio which obtained by dividing the fluorescence intensity of the positive or negative serum sample by the cutoff value(average of the negative fluorescence intensity / fluorescence intensity of the negative control = 1) as shown in Fig. 13. The protein chip analysis for HIV exhibited a very high sensitivity with the result that the S/CO ratio of the positive serum was at least 5 even at 2-8, while that of the negative serum was below the cutoff even at 2-1 and thereafter. Further, the

protein chip analysis for HCV also exhibited a high sensitivity with the result that the positive serum showed positive reaction up to 2-7, while the negative serum was below the cutoff even at 2-1 and thereafter. The result of the ELISA analysis for HIV was different from that of the protein chip; the S/CO ratio of the positive serum was at least 5 even at 2-8, as in the protein chip analysis, while that of the negative serum was above the cutoff up to 2-5. In case of HCV, the positive serum showed a gentle decrease depending on the dilution factor, while the S/CO ratio of the negative serum was above the cutoff up to 2-5. This result showed that there may be an error in determining the infection of a disease by using the ELISA method.

The results of Examples 1 to 5 demonstrated that the reactions between antigen and antibody are conducted with a sufficient effectiveness on the protein chip at room temperature. Further, as shown in Examples 6 to 8 above, it was proved that the antigen-antibody reaction using the protein chip of the present invention was superior to that in ELISA method in terms of specificity and sensitivity, considering the reaction time and temperature.

As described above, the diagnostic system using the protein chip of the present invention requires the smaller amount of antigen and antibody than those of conventional ELISA or CLIA method and provides excellent results in terms of sensitivity and specificity. Further, the inventive process is very economical in aspects of examining procedure and cost due to the reduced reaction steps and time.

What is claimed is:

1. A protein chip for mass-diagnosis or analysis of test samples, which comprises a micro solid substrate on which a plurality of spots of probe proteins are fixed in a defined arrangement, wherein,

1) the probe proteins are selected from the group consisting of antigens, receptors and enzymes;

2) the probe proteins are fixed on the micro solid substrate via bonds between amino groups of the probe proteins and functional groups of chemicals coated on the substrate;

3) the probe proteins are capable of binding to target proteins in the test samples; and

4) the quantity of the probe proteins per spot is 0.1pg or more.

2. The protein chip of claim 1, wherein the probe proteins are antigenic proteins originated from animals, plants or unicellular organisms including viruses, bacteria and fungi.

3. The protein chip of claim 2, wherein the antigenic proteins originate from Hepatitis B virus (HBV), Human immunodeficiency virus (HIV) or Hepatitis C virus (HCV).

4. The protein chip of claim 1, wherein the micro solid substrate is made of glass, modified silicone or polymer such as polystyrene, tetrafluoroethylene and polypropylene; and, the surface of the substrate is coated with a chemical selected from the group consisting of polymers, plastics, resins, carbohydrates, silica, silica derivatives, carbons, metals, inorganic glasses and membranes.

5. The protein chip of claim 1, wherein the functional group of the chemical coated on the substrate is an alkyl group.

6. The protein chip of claim 5, wherein the chemical coated on the substrate is aminoalkylsilane.

7. The protein chip of claim 1, wherein the spots of the probe proteins are circular in shape and 150~1000㎛ in diameter.

8. The protein chip of claim 1, wherein the quantity of the probe proteins per spot ranges from 1 to 100pg.

9. The protein chip of claim 1, wherein the probe proteins are fixed on the micro solid substrate by the steps of: arraying the probe proteins, previously diluted with a coating buffer, on the substrate in a defined arrangement; immersing the substrate in 100% ethanol; and drying the substrate.

10. The protein chip of claim 9, wherein the coating buffer is sodium phosphate buffer or sodium carbonate buffer.

11. The protein chip of claim 10, wherein the coating buffer is 10mM sodium phosphate buffer or 50mM sodium carbonate buffer.

12. The protein chip of claim 1, wherein the micro solid substrate is a tetragonal plate on which the protein spots are arranged in plural columns and rows or a circular disc plate on which the protein spots are arranged around the circumference.

13. The protein chip of claim 1, wherein the substrate is divided into one or more sectors and each sector has spots containing a same kind of proteins different from those in other sectors.

14. A method for manufacturing the protein chip of claim 1, which comprises the steps of:

1) arraying mixtures of a coating buffer and one or more kinds of probe proteins at predetermined locations on a micro solid substrate, with the quantity of the proteins per spot of 0.1pg or more;

2) immobilizing the probe proteins by incubating the substrate at room temperature;

3) fixing the probe proteins on the substrate by immersing the substrate in 100% ethanol; and

4) drying the substrate obtained in 3).

15. The method of claim 14, wherein the probe proteins of step 1 are selected from the group consisting of antigens, receptors and enzymes which originate from animals, plants or unicellular organisms including viruses, bacteria and fungi.

16. The method of claim 15, wherein the antigens originate from Hepatitis B virus (HBV), Human immunodeficiency virus (HIV) or Hepatitis C virus (HCV).

17. The method of claim 14, wherein the coating buffer of step 1 is sodium phosphate buffer or sodium carbonate buffer.

18. The method of claim 14, wherein the micro solid substrate of step 1 is made of glass, modified silicone or polymer such as polystyrene, tetrafluoroethylene and polypropylene; and, the surface of the substrate is coated with chemicals selected from the group consisting of polymers, plastics, resins, carbohydrates, silicas, silica derivatives, carbons, metals, inorganic glasses and membranes.

19. The method of claim 18, wherein the micro solid substrate is coated

with aminoalkylsilane.

20. The method of claim 14, wherein step 1 contains the steps of dividing the substrate into one or more sectors and arraying the probe proteins on the substrate so that each sector has proteins different from those on other sectors.

21. The method of claim 14, wherein step 1 is performed by an automatic microarrayer system.

22. A method for analyzing target proteins present in test samples quantitatively or qualitatively, which comprises the steps of:

1) reacting a test sample with the protein chip of claim 1 or 12; washing the protein chip;

2) reacting the protein chip obtained in 2) with fluorescence-conjugated secondary antibodies specific for a target protein, said target protein being capable of binding a probe protein fixed on the protein chip; and

3) detecting the reaction signals with a fluorescence microscope or a microchip reader.

23. The method of claim 22, wherein the protein chip has antigenic proteins relating to two or more diseases fixed in divided sectors thereon so that each sector contains proteins different from those on other sectors, the test sample is serum of a subject, and the reaction signals detected with a fluorescence microscope or a microchip reader refer to diagnostic indications for the diseases in the subject.

24. The method of claim 22, wherein the protein chip has antigenic proteins relating to a disease, the test samples are sera of two or more subjects, and the reaction signals detected with a fluorescence microscope

or a microchip reader refer to diagnostic indications for the disease in the subjects.

25. The method of claim 22, wherein the protein chip has antigenic proteins relating to two or more diseases, the test samples are sera of two or more subjects, and the reaction signals detected with a fluorescence microscope or a microchip reader refer to diagnostic indications for the diseases in the subjects.

26. The method of claim 23 or 25, wherein the antigenic proteins relating to two or more diseases are two or more antigenic proteins selected from the group consisting of antigenic proteins of Hepatitis B virus (HBV), Human immunodeficiency virus (HIV) and Hepatitis C virus (HCV).

27. The method of claim 22, wherein the reaction of step 3 is performed by an automatic microarrayer system.

28. The method of claim 22, wherein the fluorescent substance conjugated with the secondary antibodies of step 3 is fluorescein isothiocyanate (FITC).

29. An automated system for diagnosing a plurality of diseases in plural subjects comprising:

1) the protein chip of claim 1;

2) the first microarrayer capable of arraying one or more probe proteins in plural spots on the protein chip;

3) the second microarrayer cotrolled to perform sequentially allotting test samples exactly to the locations at which the probe proteins are fixed on the protein chip, washing the protein chip after reaction, and adding secondary antibodies to react with target proteins in the test samples; and

4) a fluorescence microscope or a micro chip reader for detecting the reaction between the probe proteins and the target proteins.

30. The system of claim 29, further comprising a computerized apparatus which is capable of compiling diagnostic data acquired by the fluorescence microscope or the micro chip reader and processing the data.

FIG. 1

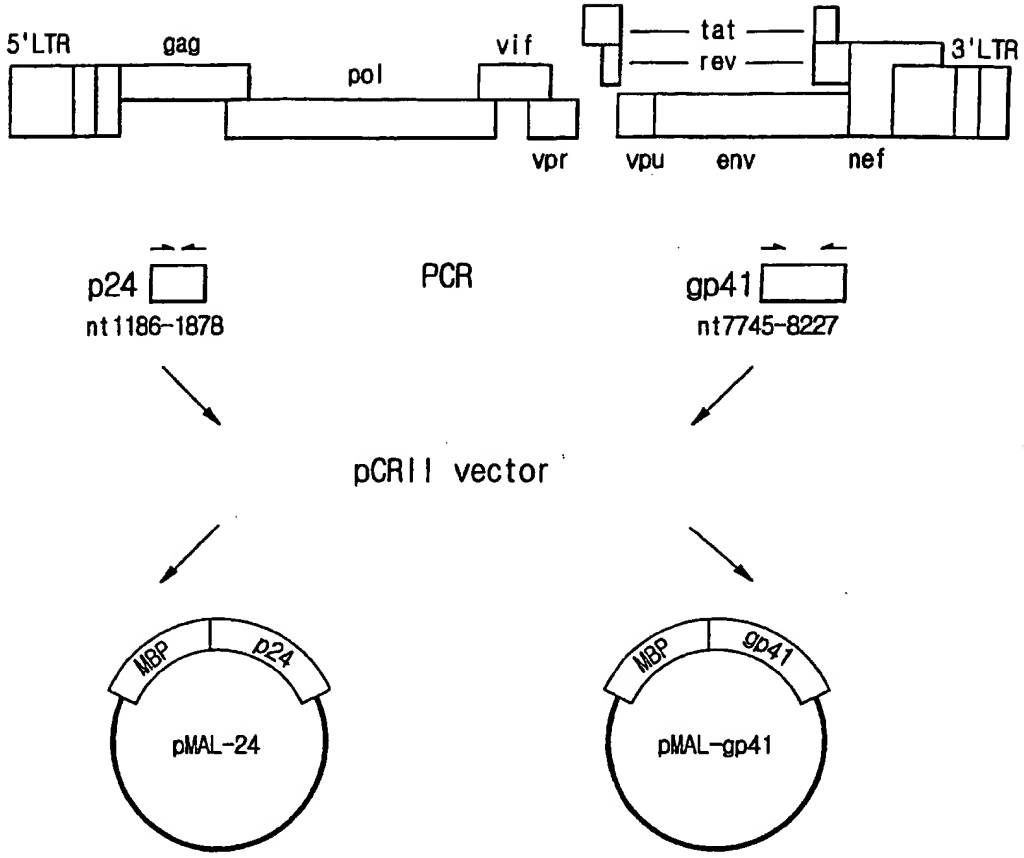

HIV-1 genome
5'LTR
gag
pol
vif
vpr
tat
rev
vpu
env
nef
3'LTR
p24
nt1186-1878
PCR
gp41
nt7745-8227
pCRII vector
MBP
p24
pMAL-24
MBP
gp41
pMAL-gp41

FIG. 2

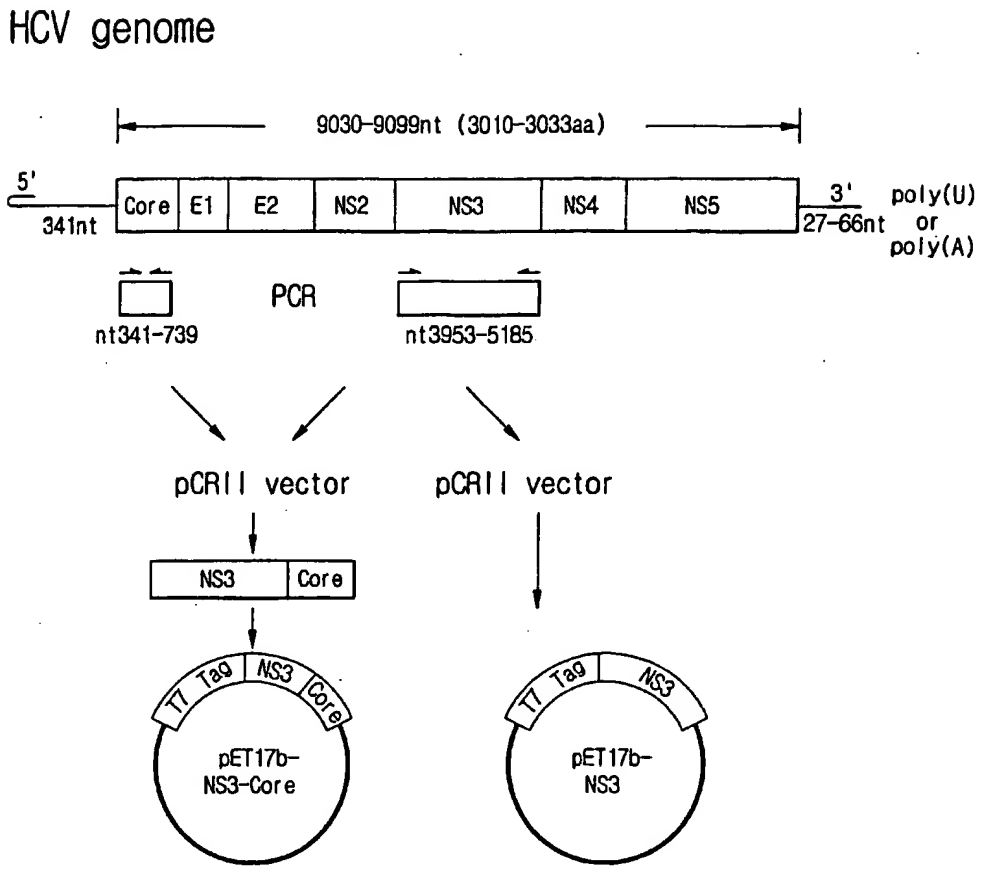

HCV genome
9030-9099nt (3010-3033aa)
5'
341nt
Core
E1
E2
NS2
NS3
NS4
NS5
3'
27-66nt
poly(U)
or
poly(A)
PCR
nt341-739
nt3953-5185
pCRII vector
pCRII vector
NS3
Core
T7
Tag
NS3
Core
pET17b-
NS3-Core
T7
Tag
NS3
pET17b-
NS3

FIG. 3

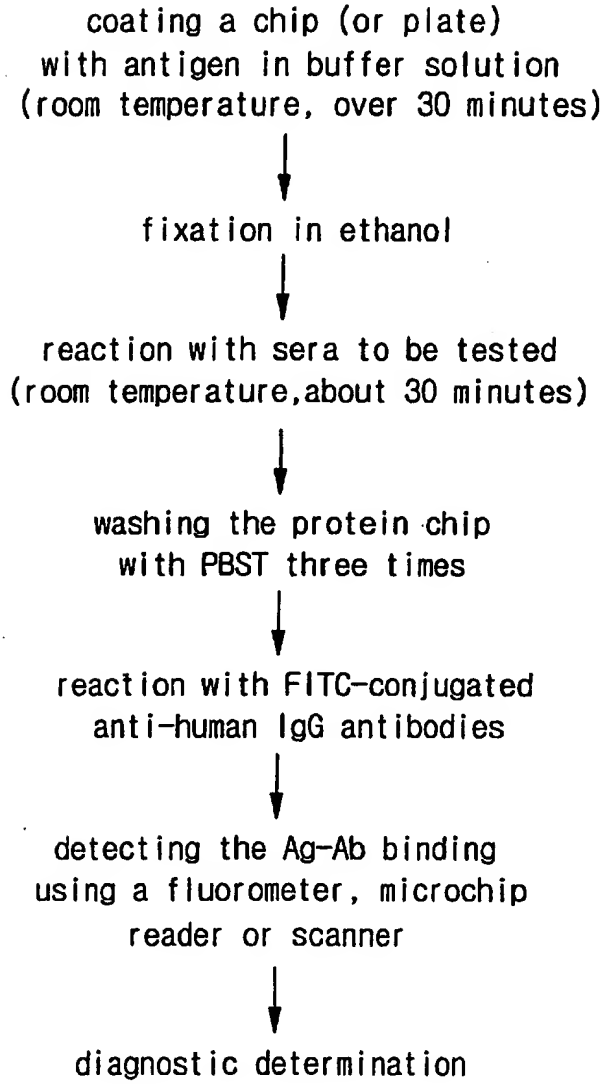

coating a chip (or plate)
with antigen in buffer solution
(room temperature, over 30 minutes)
fixation in ethanol
reaction with sera to be tested
(room temperature,about 30 minutes)
washing the protein chip
with PBST three times
reaction with FITC-conjugated
anti-human IgG antibodies
detecting the Ag-Ab binding
using a fluorometer, microchip
reader or scanner
diagnostic determination

FIG. 4a

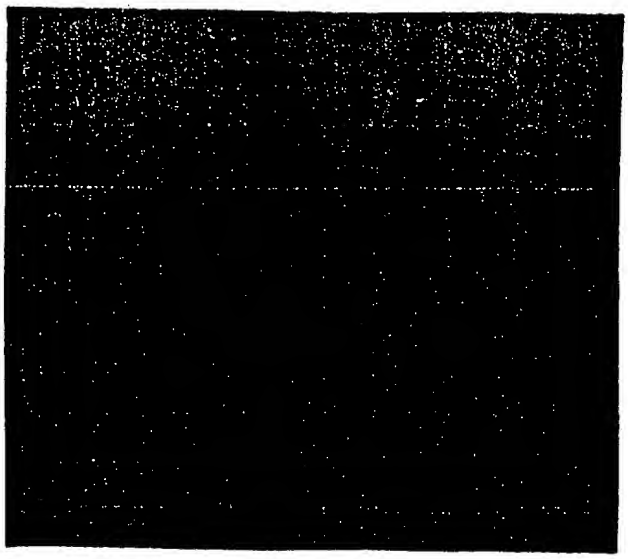


FIG. 4b

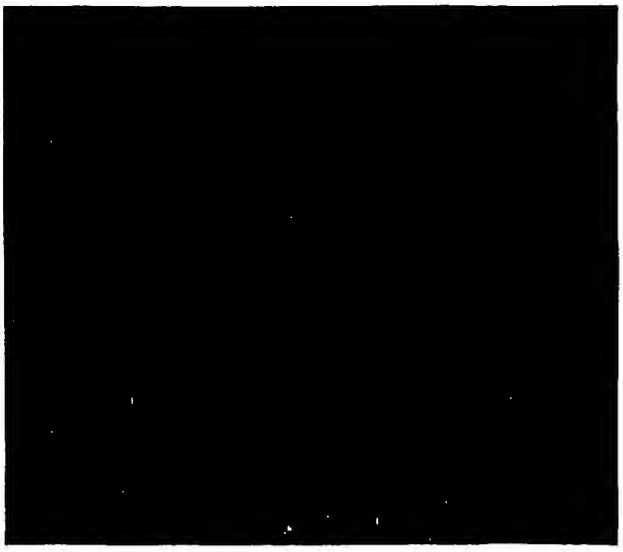


FIG. 5a

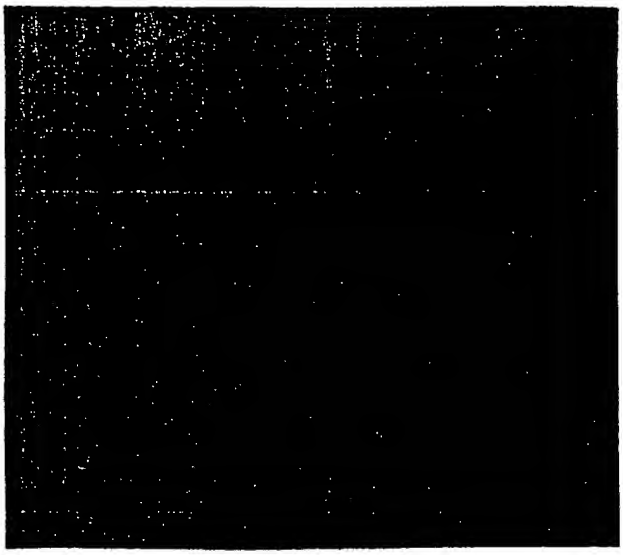


FIG. 5b

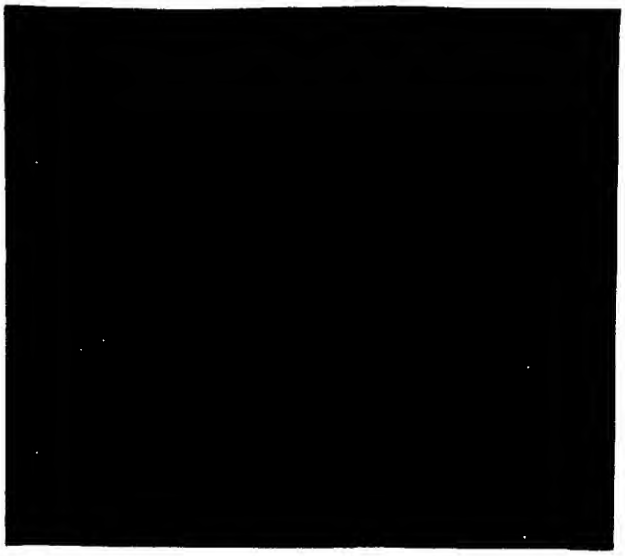


FIG. 6a

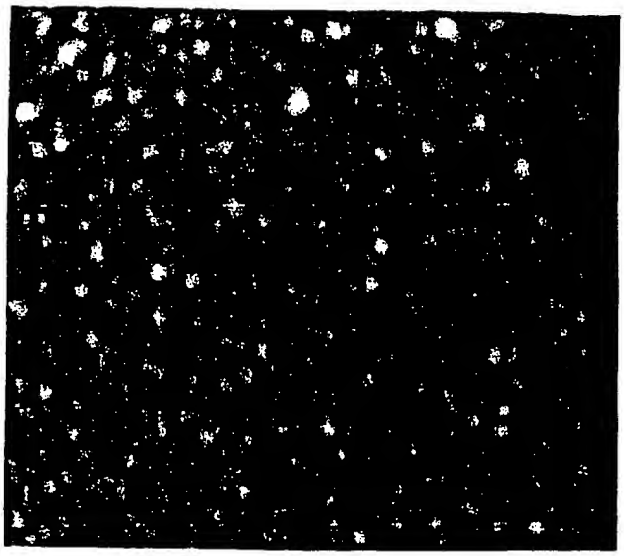


FIG. 6b

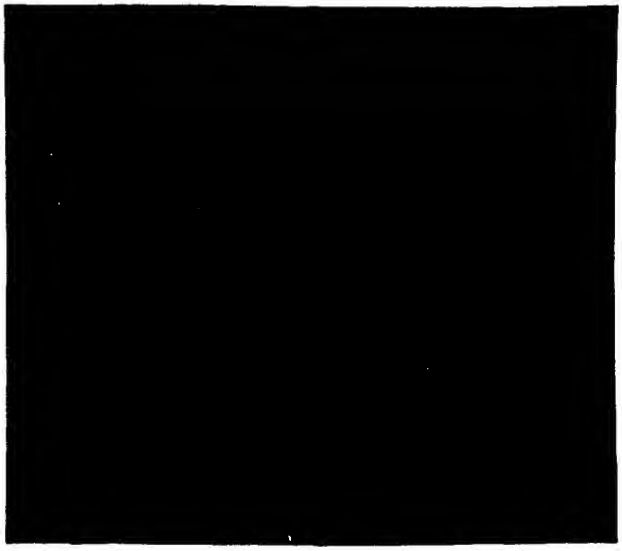


FIG. 7

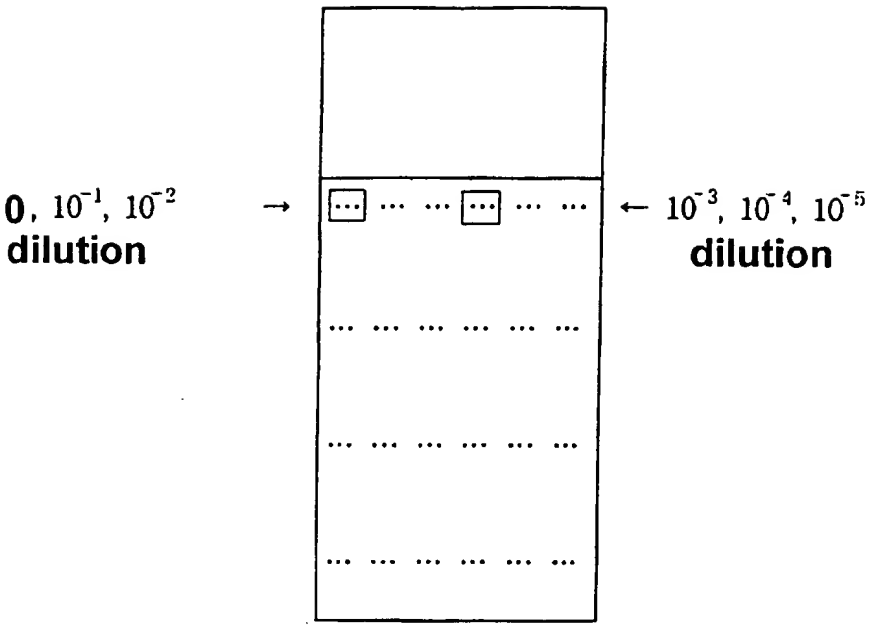


FIG. 8

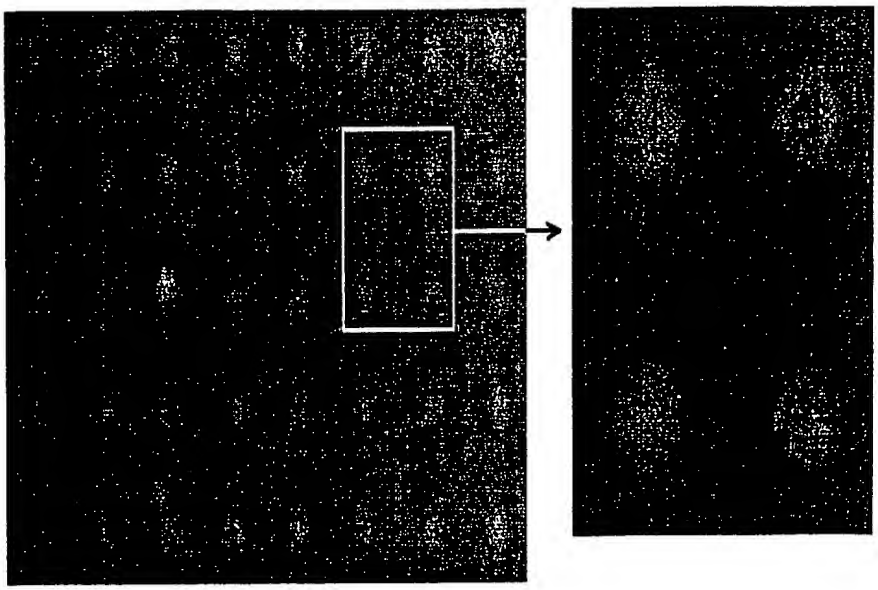


FIG. 9

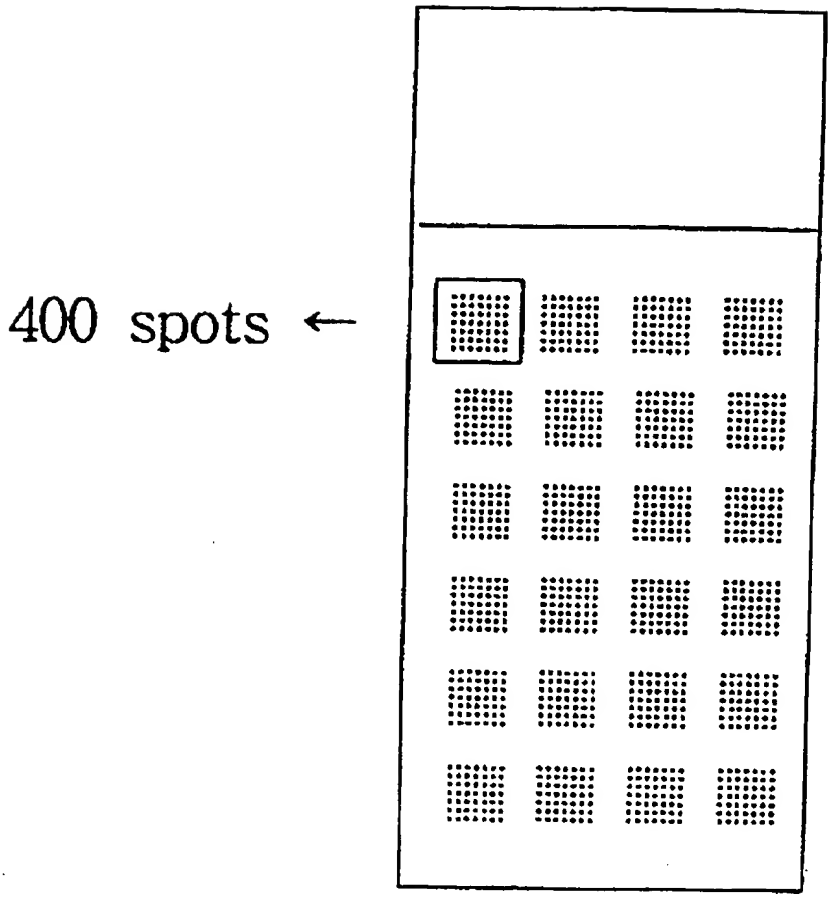


FIG. 10a

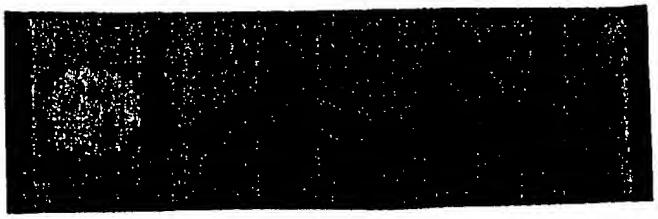


FIG. 10b

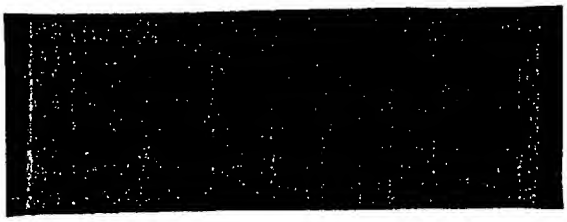


FIG. 11a

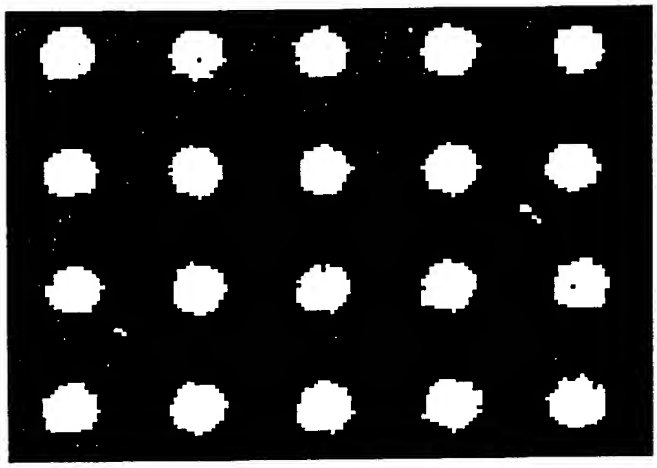


FIG. 11b

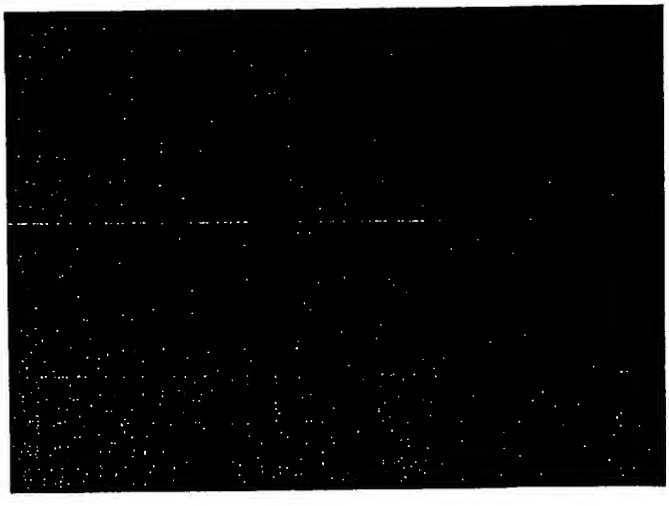


FIG. 12a

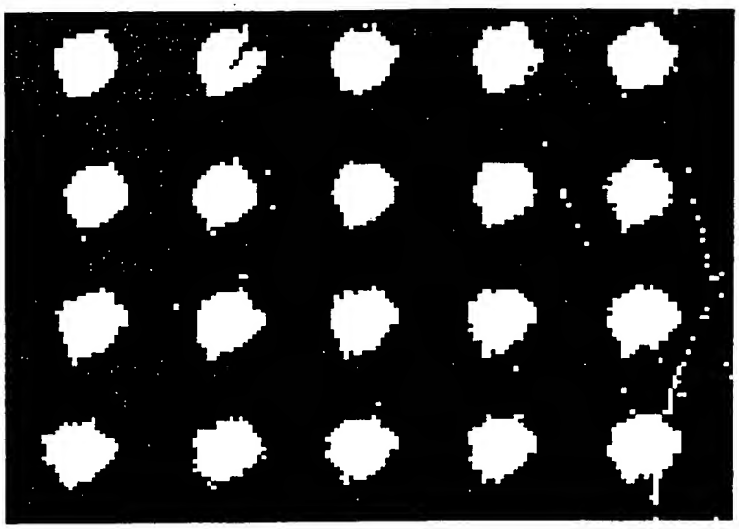


FIG. 12b

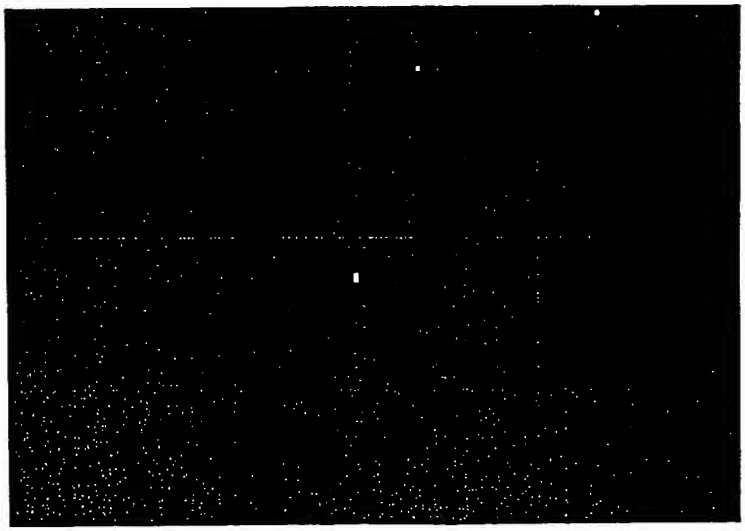
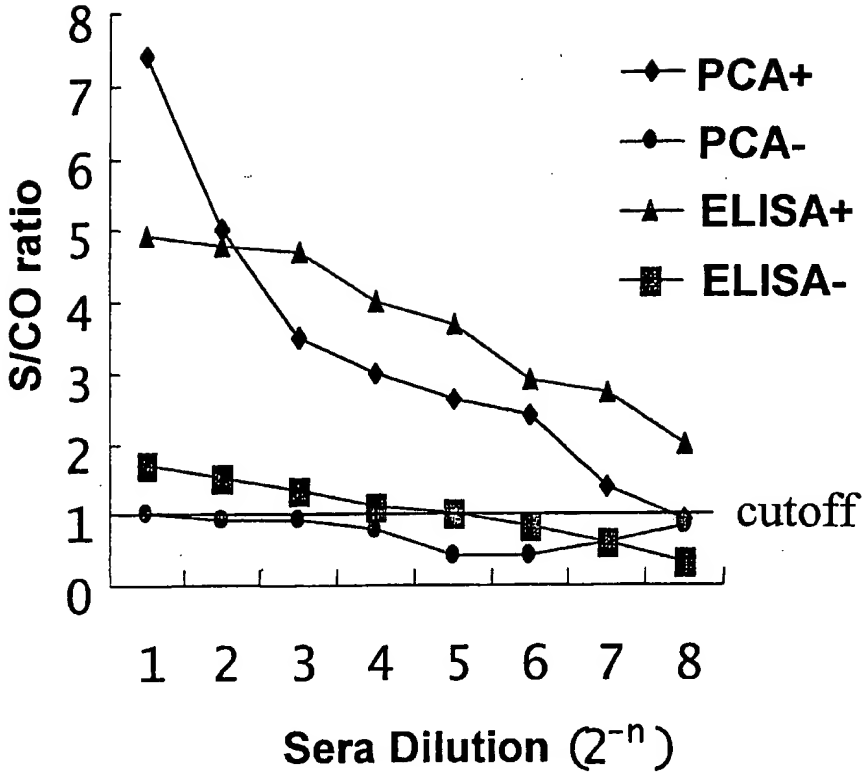


FIG. 13a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4
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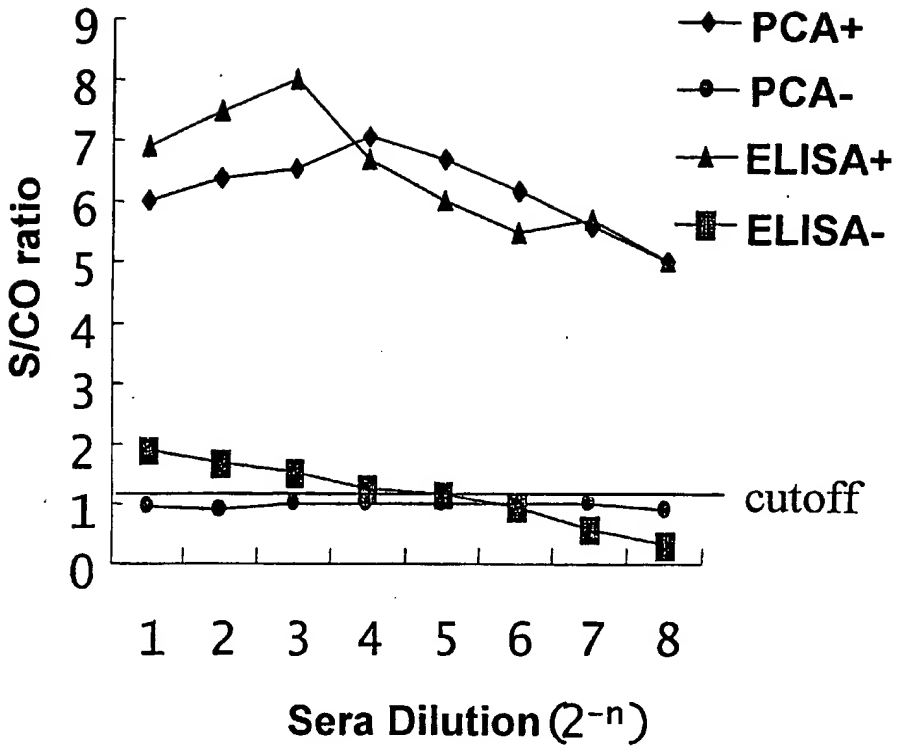
PCA-
ELISA+
ELISA-
cutoff

FIG. 13b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PCA-
ELISA+
ELISA-
cutoff
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Sera Dilution (2-n)

SEQUENCE LISTING

<110> KIM, Sun-Young

<120> Multipurpose diagnostic systems using protein chips

<130> OPF0065/PCT

<150> KR 99-34427
<151> 1999-08-19

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00928

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C07K 17/00, G01N 33/53, G01N 33/533, G01N 33/533

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC7 C07K 17/00, G01N 33/53, G01N 33/533

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Korean patents and applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)

PubMed, CASLINK(STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 98/49557 A (B-E SAFE, INC) 5 NOVEMBER 1998	1 - 30
X	EP 0818467 A2 (NEC CORP.) 14 JANUARY 1998	1 - 30
X	LUEKING ET AL. 'PROTEIN MICROARRAYS FOR GENE EXPRESSION AND ANTIBODY SCREENING' IN:ANAL BIOCHEM, 1999, VOL.270, P103-11	1 - 30
X	US 5,591,646 A (ARRIS PHARMACEUTICAL) 7 JANUARY 1997	1 - 4
P,X	HOLT ET AL. 'BY-PASSING SELECTION: DIRECT SCREENING FOR ANTIBODY-ANTIGEN INTERACTIONS USING PROTEIN ARRAYS' IN:NUCLEIC ACIDS RES 1 AUGUST 2000, VOL.28, PE72	1 - 30
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☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 DECEMBER 2000 (12.12.2000)	13 DECEMBER 2000 (13.12.2000)

Name and mailing address of the ISA/KR	Authorized officer
Korean Industrial Property Office Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea	HAN, Hyun Sook
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5596

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR00/00928

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 98/49557	05.11.98	AU 7164998	24.11.98
EP 0818467	14.01.98	JP 10025299	27.01.98